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Zoological Research

Phylogenetic Relationships of 11 Bumblebee Species (Hymenoptera: Apidae) Based on Mitochondrial Cytochrome *b* Gene Sequences

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Abstract: Phylogenetic relationships of 11 bumblebee species, including 5 subgenera: *Bombus* (5 species), *Thoracobombus* (3 species), *Mendacibombus* (1 species), *Fervidobombus* (1 species) and *Pyrobombus* (1 species), were analyzed based on the 357 bp mitochondrial cytochrome *b* gene sequences. There are 65 singleton polymorphic sites and 71 parsimony informative polymorphic sites in this DNA segment, and 45 polymorphic sites within the total 119 translated amino acids segment. Both NJ tree and MP tree show that *Mendacibombus* (*B. avinoviellus*) is basal to others, followed by *Fervidobombus* (*B. pensylvanicus*); *Pyrobombus* (*B. impatiens*) and *Bombus* are sister subgenera; the subgenus of *Bombus* is monophyletic, in which *B. ignitus* diverged first.

Key words: *Bombus*; Cytochrome *b* gene; DNA sequence; Amino acid sequence; Molecular phylogeny

细胞色素 *b* 基因序列与 11 种熊蜂的系统进化

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摘要: 通过比较一段 357 bp 的细胞色素 *b* 的序列, 分析了熊蜂属 5 亚属 11 种熊蜂系统发育关系。5 亚属包括 *Bombus* (5 种)、*Thoracobombus* (3 种)、*Mendacibombus* (1 种)、*Fervidobombus* (1 种) 和 *Pyrobombus* (1 种)。该序列有 65 个单变异多态位点和 71 个简约信息多态位点, 翻译成 119 个氨基酸序列后有 45 个氨基酸变异位点。根据 P - 距离构建的邻接树 (NJ tree) 和最大简约树 (MP tree) 都显示同样的结果: *Mendacibombus* (*B. avinoviellus*) 分化最早; *Fervidobombus* (*B. pensylvanicus*) 次之; *Pyrobombus* (*B. impatiens*) 和 *Bombus* 形成姊妹群; *Bombus* 亚属是单系群, 其中 *B. ignitus* 在所研究的 5 个种中分化最早。

关键词: 熊蜂属; 细胞色素 *b* 基因; DNA 序列; 氨基酸序列; 分子系统发育
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There are 239 bumblebee species recognized in the world (Williams, 1998), but the phylogenetic relationships among them are still poorly understood because of their "monotonous" morphology (Michener, 1990), especially within subgenus, such as *Bombus* (Williams, 1991). So some researchers have turned to molecular markers (Stephen & Cheldelin, 1973; Pekkarinen *et al.*, 1979; Pamilo *et al.*, 1987; Pedersen, 1996; Koulianos, 1999; Koulianos & Schmid-Hempel, 2000). However, in *Bombus s.*

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str the molecular results (Pamilo *et al*., 1987; Pedersen, 1996) did not concur with morphology results (Plowright & Stephen, 1973; Williams, 1994). The phylogenetic relationships of those bee species are different from author to author (Sakagami, 1976; Williams, 1994; Pedersen, 1996; Koulianos & Schmid-Hempel, 2000).

Mitochondrial DNA (mtDNA) is a good genetic marker because of its high mutation rate and haploid without recombination (Brown *et al*., 1979; Avise *et al*., 1987; Moritz *et al*., 1987). The honeybee's complete mtDNA genome was sequenced (Crozier & Crozier, 1993), which makes the genetic analysis of bees much easier.

There are 10 species in subgenus of *Bombus*, and most of them distribute in Asia (Williams, 1998). Here we sequenced cytochrome *b* gene to study the phylogenetic relationships among 11 species including subgenus *Bombus* and four other subgenera with *Apis mellifera* as the outgroup.

1 Materials and Methods

1.1 Materials and PCR sequencing

Bumblebees were collected from Beijing (*Bombus ignitus* and *B. hypocrita*), Jilin (*B. patagiatus*) and Zhejiang (*B. ignitus*) in China, Korea (*B. ignitus*) and Japan (*B. ignitus*) during 2000 and 2001. The samples were kept in pure alcohol at room temperature. Total DNA from 16 *B. ignitus*, 4 *B. hypocrita* and 2 *B. patagiatus* was extracted from the heads, thorax (Garnery *et al*., 1991) or legs (Koulianos, 1999) using the standard phenol/chloroform methods.

Partial cytochrome *b* gene amplified with the primer CB1 and CB2 (Simon *et al*., 1994) coordinates 11 400 – 11 425 and 11 859 – 11 884 as described in Crozier & Crozier (1993). The reaction mixture consisted of 10 – 20 ng total DNA, 2 μL mixed dNTP (4 mmol/μL), 1 μL each kind of primers (10 pmol/μL), 3 μL $MgCl_2$ (25 mmol/μL), 5 μL buffer (10 ×, 500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 9.0, Triton X – 100), 2 μL BSA (bovine serum albumin) and 2 U Taq DNA polymerase, then adding ddH_2O to total 50 μL. After an initial denaturation at 96 ℃ for 3 min, 35 cycles were performed with denaturing at 96 ℃ for 1 min, annealing at 54 ℃ for 1 min and extension at 72 ℃ for 1 min, followed by an end extension for 5 min at 72 ℃. The PCR products were sequenced with BigDye™ Terminator cycle sequencing kits (Perkin-Elmer Biosystems) using ABI377 automatic sequencer (PE). Each individual was sequenced in both directions. The sequences were checked by eyes and edited with DNAstar (DNASTAR Inc.1996) package. Cytochrome *b* gene sequences GenBank accession numbers of *B. hypocrita*, *B. ignitus* and *B. patagiatus* are AF498397, AF498398 and AF498399.

1.2 Sequences from GenBank

The sequences in 8 bumblebee species of 5 subgenera and *Apis mellifera* were collected from GenBank (Table 1).

Table 1 Sequence data from Genbank in bumblebee and honeybee species

Subgenus	Species	No. of sequence
Bombus	*B. lucorum*	AF002722
	B. terrestris	AF002721
Thoracobombus	*B. pascuorus*	AF017516
	B. humilis	AF017517
	B. ruderarius	AF002723
Mendacibombus	*B. avinoviellus*	AF181610
Fervidobombus	*B. pennsylvanicus*	AF181611
Pyrobombus	*B. impatiens*	AF281169
	Apis mellifera	L06178

1.3 Phylogenetic analysis

The polymorphic sites and synonymous/non-synonymous substitutes were calculated by software DNASP 3.0 (Rozas & Rozas, 1999). We use the amino acid sequence to construct the phylogenic trees. NJ tree was constructed with MEGA version 2.1 (Kumar *et al*., 2001) based on P-distance (Nei & Kumar, 2000), while maximum parsimony tree with PAUP*4.08b (Swofford, 2001) by heuristic search with TBR branch swapping. *Apis mellifera* was used as the outgroup. For both NJ and MP tree, we used 2000 bootstrap replications to estimate the support rate. We reconstructed NJ tree of subgenus *Bombus* based on DNA sequence to further analyze the phylogeny, because within subgenus DNA sequence can reflect the relationship better than amino acid sequence.

2 Results and Discussion

There are 65 singleton polymorphic sites and 71 parsimony informative polymorphic sites in this DNA segment; 45 polymorphic sites within the total 119 translated amino acids segment; but no gap or terminal code were found (Fig.1a, b). The mutation pattern of the segment suggests that it is probably from mitochondrial genome, not from nuclear psuedogene. Because the mean transition/transversion is 0.6347 and the rate of amino acid polymorphic site is high (37.82%), we used the amino acid sequence to construct the phylogenetic trees. Both NJ and MP trees showed the same pattern: *Mendacibombus* (*B. avinoviellus*) is basal to others, followed by *Fervidobombus* (*B. pensylvanicus*); *Pyrobombus* (*B. impatiens*) and *Bombus* are sister subgenera (Fig.2a, b). The subgenus of *Bombus* is monophyletic: *B. locrum*, *B. pat*-

a

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                                        111111111 1111111111 1111111111 1111111111
         1111222 3333444455 5555577889 9011112233 3344444555 5566666777 7778888999
       3592589124 0136568901 3478925142 3802477925 6824578134 7935689012 4780369258
1)     ATTTTTTAAT AATAACATTT CATGAAAATA TTATATTAGC ATTAAAAGAT AATAATATTC TCATAAAATA
2)     T.A.....G. C...T..... TT.......C .C.A..CTT. .........C T.CT.A...T AT..TT...T
3)     ..A.A..... .TATT..... .......T.. ........AT ..CT..T... .T...A...T AT...TTT.T
4)     ..ACA..... .TATT..... .......T.. ........AT ..CT..T.T. .T...G...T AT.A.TT..T
5)     T.A.ACCT.. T...T..... .T........ C...T...AT ....T..... .T...A...T AT...TT...
6)     ....A..... ...T...... .....T.... ........AT ..CTT.T.T. .T...AG..T AT....T..T
7)     ..ACA..... ....T..... .T......C. ........AT ....T..... .....A...T A....TT.CT
8)     ..A.A..... .TA.T..... .....CTT.. ........AT ..CT..T.T. .T...A...T AT...CT..T
9)     ..A.A.CT.. ....T..... TT........ ......C..AT ....T..A.. .T...A..CT AT...T....
10)    T...A.CT.. ....C..... .T...G.... ....T...AT ....T..... .T...A...T AT...TT...
11)    T...A.CT.. ....T..... .T........ ....T...AT ....T..... .TC..A...T AT...TT...
12)    .C....CTTA ...TTGTACA TTATTT...C ..T.TA..AA TA..TT..TA CT..T.GCCT ATTAT..T.T

       1222222222 2222222222 2222222222 2222222222 2222233333 3333333333 333333
       9000001111 1223333444 4456666666 7777777888 8889900011 1111222233 445555
       9123470136 8890147036 7991245678 0124689234 5685746902 5789015736 281457
1)     ATCATAATAT TTAACTCATT AATAATACAA CATTACATAT TTATTTAAAA TATATTATAT TGATCA
2)     TA.......C ......T... TT......T. T....TT.T. AATA....TT C..T.A.AT. .A.C..
3)     .A.....A.. ....T.TT.. .......... A.......TC AA.A...GTT G..G..GA.. .A..T.
4)     .......A.. ....T.TT.. .......... T...T...TC AA.A.CT.TT A.....GA.. .A..TT
5)     .......A.. ....T.T..C .T......T. T...T..... AAT.A...T. .T...G.AT. .A...T
6)     C....T.AT. ....T.T... T......... T..A.T..T. A..A.CT.TT A....AGA.. .A..TT
7)     .C.....A.. .C..TCT..C .T......T. T...T...TC AA..A...T. .T...AGAT. .A...T
8)     .......A.. ....T.TT.. .......... T...T...TC AATA.CT.TT A.....GGG. .A..TT
9)     .......A.. ....T.T... .T........ A...C...TC A....C.... ......A.AT. CAT..T
10)    .......A.C ....TCT.CC .T......T. T...T..... AAT.A...T. .TC..A.A.. .A...T
11)    .......A.C ....TCT.CC .T......T. T...T..... AAT.A...T. .TC..A.A.. .A...T
12)    C.GGA.T.T. A.TCA.T... T.CTTAGG.T TTAACTCAT. .AC.C.T.TT A..CA.GA.C CT....
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b            1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
    1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
1)  AMVITNLISA IPYIGQFTVE WIWGGFSINN DTLNRFYSFH FILPFIILMM VFMHLIILHI TGSSNPIHSK
2)  .......V.. .......I.. .......... A......T.. .......... .LL..M.... ......L...
3)  .......... L......... .......... .......... .........L ..I..M.... ......M...
4)  .......... L......... .......... .......... .........L ..I..M...M ..........
5)  .......... .......... .......... .......... ........L. ..I..M.... ..........
6)  .......... .......... .......... .......... ........LL ..I..MV... ......L...
7)  .......... .......... .......... .......... ........L. .....M.... ..........
8)  .......... L......... .......... .......... .........L ..I..M.... ..........
9)  .......... .......I.. .......... .......... ........L. M.I..M.... ..........
10) .......... .......... .......... .......... ........L. ..I..M.... ..........
11) .......... .......... .......... .......... ........L. ..I..M.... ..........
12) .T.....L.. .....DTI.L .......... A.....F.L. ....LL..F. .IL..FA..L ......LG.N

                                 1 1111111111 111111111
    7777777778 8888888889 9999999990 0000000001 111111111
    1234567890 1234567890 1234567890 1234567890 123456789
1)  LNIYKINFHP YFTIKDLITI ILTFILFMFI NLQMPYILSD PDNFKMANP
2)  .......... ..S....... ..I.LI..I. ...F?.L... .........
3)  M......... .........M ....SM.I.. ...F..V.G. ........S
4)  M......... .......... .F..SM.I.. ...F....G. ........S
5)  M......... .......... .F..MI.L.. ...L.FM... .........
6)  I......... ..S....... .MM.L..I.. ...F..M.G. ........S
7)  M......... .......... .F..SM.L.. ...L.FM.G. .........
8)  M......... .......... .F..SI.I.. ...F....G. ........S
9)  M......... .........M .F..S..... ......M... .........
10) M......... .......... .F..MI.L.. ...L.FM... .........
11) M......... .......... .F..MI.L.. ...L.FM... .........
12) F.N..S.... ..S....LGF YIILFI.... .F.F..H.G. .....I...
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Fig.1 Segment mutation sites of cytochrome *b* DNA and amino acid sequence in 12 bumblebee and honeybee species

a. 357-segment cytochrome *b* DNA sequence; b. 119-segment cytochrome *b* amino acid sequence.

1) *B. impatiens*, 2) *B. avinoviellus*, 3) *B. pascuorus*, 4) *B. humilis*, 5) *B. locrum*, 6) *B. pennsylvanicus*, 7) *B. terristris*, 8) *B. ruderarius*, 9) *B. ignitus*, 10) *B. hypocrita*, 11) *B. patagiatus*, 12) *Apis mellifera*.

Figure above those sequence sites is the site position of the segment sequence; dotes indicate the nucleotides identical to that of *B. impatiens*.

agiatus and *B. hypocrita* are closely related to each other, and *B. ignitus* diverged first among those five species (Fig.2a, b). MP tree length is 83, Consistency Index is 0.819, Retention Index is 0.712 and Rescaled Consistency Index is 0.583. Similar results for the subgenus of *Bombus* were obtained using DNA sequence directly, except that *B. patagiatus* and *B. hypcrita* become the most derived sister species.

The phylogeny of bumblebees has not been resolved yet because of "monotonous" morphology (Williams, 1991). Now by the analysis of the mitochondrial cytochrome *b* sequences, we provided new

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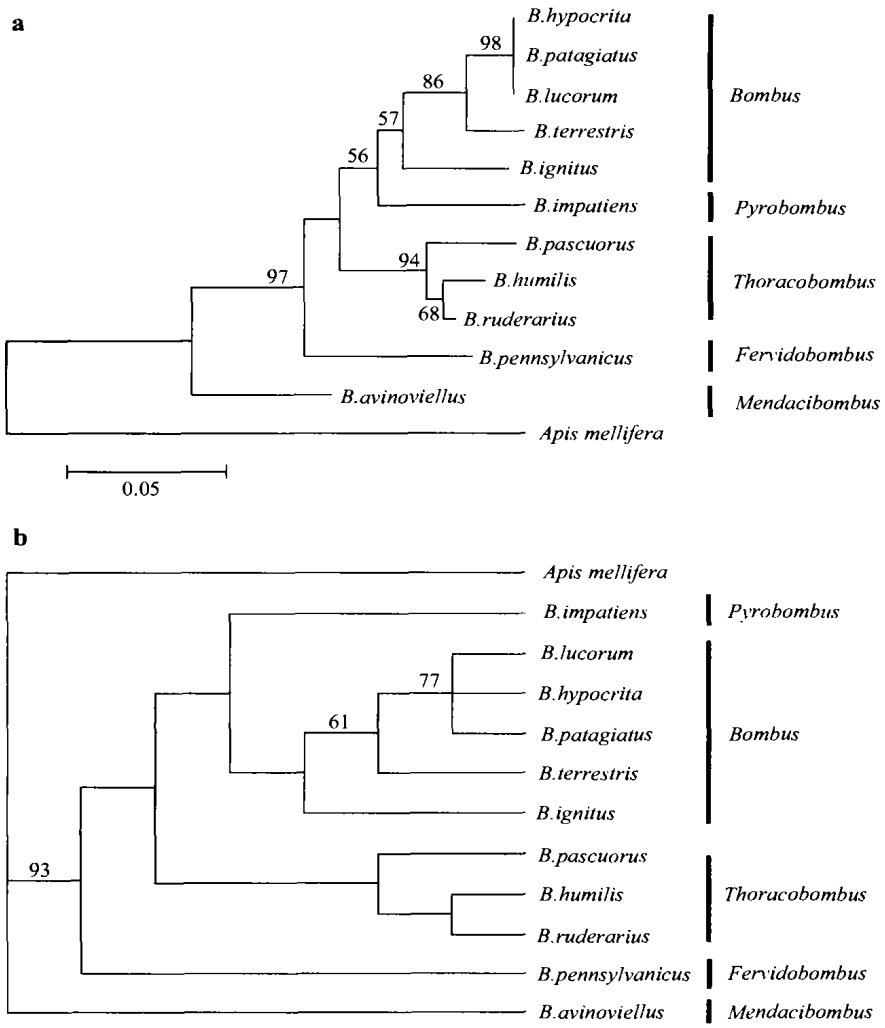

Fig.2 Phylogenetic trees of 11 bumblebee species based on mitochondrial cytochrome *b* gene sequences

a. NJ tree constructed with MEGA V2.1 based on P-distance; b. Topology from MP analysis using PAUP* V4.08b.

The numbers at the nodes are bootstrapping indices of 2 000 replicates of the level of support for individual nodes, and data are dismissed when the support rate is less 50%. The corresponding subgenera are shown at the right.

information on phylogenetic relationship of bumblebees.

The topology of subgenera from our molecular data is similar to that of Williams' morphologic study (Williams, 1994), but differs from other recent molecular studies. Pedersen (1996) showed that *Bombus* was basal to *Pyrobombus* and several other subgenera on the basis of COI sequence. Even based on the same COI sequences, the results of Koulianos & Schmid-Hempel (2000) differed from that of Pedersen (1996). The former results indicated that *Bombus* and *Pyrobombus* were paraphyletic between two clades, but the latter ones showed that *Bombus* was basal to *Pyrobombus* in a monophyletic clade. The result based on cytochrome *b* sequences was similar to that of Pedersen (1996) for the two subgenera (Koulianos & Schmid-Hempel, 2000). However, all the studies shared some similar patterns that the subgenus *Bombus* is monophyletic.

Because of the high rate of polymorphic sites among our analysis data, we believe that mutation of mtDNA among subgenera is almost saturated, so the

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translated amino acid sequence is better than DNA sequence for the phylogenetic analysis of bumblebee subgenera. In the future, it is necessary to study more species using other more conservative genes.

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